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The origin of follicular bile acids in the human ovary

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Abstract

Bile acids (BA) are present in ovarian follicular fluid (FF) and have been linked to embryo development. However, information on the source of ovarian BA is scarce. Therefore, we aimed to explore local ovarian synthesis and BA transport from blood into FF. BA levels were determined in matching FF and serum from women who underwent *in vitro* fertilization. *In vitro* BA production by human mural (MCG) and cumulus granulosa cells (CGC) was measured by mass spectrometry. Gene and protein expression were quantified in human MCG and CGC and in human ovarian tissue by quantitative PCR and Western blot/immunohistochemistry, respectively. There was a significant correlation between the levels of BA in blood and FF (r_s=0.186, P = 0.027). Interestingly, levels of FF BA were almost double those in blood (P < 0.001) with a higher percentage of primary BA, indicating that, in addition to passive diffusion, other sources of ovarian BA might exist. The key BA synthesis enzyme CYP7A1 was absent in MGC and CGC, and there was no evidence of BA production *in vitro*. Therefore, local ovarian BA production is unlikely. However, common BA importers (NTCP, ASBT) and an exporter (ABCC3) were identified in GC, theca cells, and the oocyte. In summary, these results suggest that passive and active transport of BA from blood into FF constitute sources of FF BA.

Introduction

Prior to ovulation, oocytes develop in ovarian follicles. Each preovulatory follicle has an external, vascularized layer of theca cells and an avascular internal space that consists of granulosa cells (GC), the oocyte, and follicular fluid (FF) [1]. GC are metabolically active cells that line the interior of the follicle (mural GC: MGC) and form a layer around the oocyte (cumulus GC: CGC). The proximity of the CGC to the oocyte is functionally important[2]. Indeed, oocyte maturation is regulated by the surrounding CGC [3], which provide most of its metabolic substrates [4, 5]. Moreover, the oocyte regulates expansion of the CGC population, which in turn has been shown to impact fertility [6]. Thus, a functioning relationship between CGC and oocyte is crucial for oocyte development.

In the growing follicle, GC contribute to the accumulation of increasing amounts of FF that surround the oocyte. FF thus constitutes the natural environment of the oocyte during maturation. Moreover, alterations in its composition have been linked to oocyte development and embryo quality [7-9]. The composition of FF is determined by two main factors: passive or active transport of metabolites from the systemic circulation and local production by GC. Passage of metabolites from blood into FF seems to be size-dependent, with small components passing freely and larger ones selectively or facilitated by transporters, if at all [1]. Therefore, alterations in the maternal blood composition of smaller molecules are expected to be to a certain extent reflected in FF, the microenvironment of the developing oocyte [10]. With regards to local production, GC are capable of modifying the composition of FF by secreting or degrading metabolites. For example, in addition to local steroid production[11], human GC were demonstrated to secrete very-low density lipoproteins (VLDL) and higher levels of FF VLDL were correlated with better *in vitro* fertilization (IVF) outcomes [12]. Thus, the final FF composition of the mature follicle is a reflection of systemic maternal and local follicular metabolism.

Bile acids (BA) are steroid compounds that are classically involved in fat and vitamin absorption in the gut, but are increasingly recognized for their endocrine roles, such as in glucose and lipid metabolism [13, 14]. BA production occurs via two pathways: the classic (neutral) and the alternative (acidic)

pathway [15]. To preserve the BA pool, loss of BA is prevented by active reuptake from the digestive tract into the blood stream via specialized transporters in the intestine and liver [16]. Importantly, BA have been shown to be present in FF and are linked to embryo development in IVF, indicating that BA may have biological relevance for oocyte maturation [8]. Hence, it is conceivable that alterations in the FF BA pool may have an impact on the oocyte and, consequently, on embryo development. However, the origin of BA in FF has not been clarified. In addition to simple diffusion from blood into FF, other sources of BA, such as local production and active transport, could play a role [17]. The present study thus aimed to delineate the origin of ovarian BA with a focus on the two possibilities, local intrafollicular synthesis and import from the blood compartment.

Materials and methods

Follicular fluid and serum collection

Follicular fluid and serum from patients undergoing modified natural cycle (MNC)-IVF at the University Medical Center Groningen (UMCG) in Groningen, the Netherlands, were collected during a multicenter cohort study on the outcomes of MNC-IVF [18]. Our group has previously reported on BA levels in a subset of these samples consisting of only samples from the first IVF cycle of a patient [8, 18]. For the present study, samples from a subsequent IVF cycle were used. Details on patient inclusion and study protocol have been previously published [8, 18]. A universal consent form was signed by all patients and their confidentiality was protected during the study by assigning patients and samples untraceable codes. None of the patients objected to the use of their so-called waste material (such as FF, GC, and surplus serum), which routinely becomes available during patient care and would otherwise be discarded. Fasted blood was drawn on the morning of oocyte retrieval and surplus serum was stored under the respective study code at -20 °C. After oocyte collection, the remaining FF was centrifuged for 20 min at 300 g and the supernatant was stored under the respective study code at -20 °C.

For the current study, FF and matching blood samples fulfilling the following criteria were used: FF was free of blood contamination upon visual inspection, no or one oocyte was retrieved, only one mature follicle was detected by ultrasound at ovum pickup.

Ethical approval from the Institutional Review Board was requested but waived, since materials were anonymized, and patients had signed the universal consent form.

Modified natural cycle – in vitro *fertilization procedure and collection of follicular fluid for bile acid analysis*

The MNC-IVF procedure has been described in detail previously [18, 19].In contrast to classic hyperstimulation IVF, where multiple dominant follicles develop in each cycle, in MNC-IVF only one dominant follicle develops and its contents are retrieved at ovum pickup. Consequently, the composition of the FF can be accurately compared to that of the matching blood sample.

In short, when the diameter of a natural growing ovarian follicle (measured by vaginal ultrasonography) reached 14 mm, daily injections of 0.25 mg GnRH antagonist and 150 IU recombinant FSH were started. When the diameter of the dominant follicle reached a minimum 18 mm and/or serum estradiol levels exceeded 0.8 nmol/L, 10 000 IU hCG was administered to prevent ovulation. Approximately 34 h later the oocyte was retrieved with a single-lumen aspiration needle and without flushing of the follicle. The oocyte was inseminated following standard procedure. If macroscopic blood contamination was absent, the FF was centrifuged for 20 minutes at 300 g and the supernatant was stored at -80 ^{o}C for later analysis.

Controlled ovarian hyperstimulation – in vitro *fertilization procedure and collection of granulosa cells*

The controlled ovarian hyperstimulation (COH)-IVF procedure has been described in detail elsewhere [20]. In short, on day 23 of the previous menstrual cycle or during the use of oral contraceptives

patients were started on a hormonal down-regulation protocol with daily subcutaneous triptorelin 0.5 mg GnRH analog and human menopausal gonadoprophin 150 to 225 IU up until the day of hCG injection. When the diameter of at least three dominant follicles reached a minimum 18 mm, 5 000 IU hCG was administered for final maturation of the follicle and oocyte. Approximately 36 h later the oocytes were retrieved with a single-lumen aspiration needle. Cumulus aggregates (CGC) were manually separated from the oocyte and used for experiments. After removal of cumulus-oocyte complexes, the FF was saved in 50 mL Falcon tubes (Corning, Reynosa, Mexico) at 37.5 ^{o}C and 5% CO_2 until they could be used for isolation of MGC later the same day. Only the laboratory technician and the fertility physician that were involved in the clinical procedure were aware of the identity and characteristics of the patient, but not the researchers.

Cumulus and mural granulosa cell isolation and culture

Primary MGC and CGC were obtained from pre-ovulatory follicles from women undergoing controlled ovarian hyperstimulation-IVF (COH-IVF) at the UMCG. CGC were manually separated from the cumulus-oocyte complex and washed once in Hank's Balanced Salt Solution (HBSS) (Life Technologies, USA) before being brought into culture or stored for further analysis. For the isolation of MGC, FF was centrifuged for 5 min at 400 g followed immediately by 5 min at 500 g. Red blood cells were removed by layering the cell pellet on a 40% Percoll (GE Healthcare, Uppsala, Sweden) solution followed by centrifugation for 10 min at 550 g. The suspension of GC was gently pipetted out and washed twice in HBSS to remove the Percoll solution. Both CGC and MGC were either stored for later analysis or brought into culture. In the latter case, both cumulus and MGC were first incubated in trypsin for 3 minutes at 37 ^{o}C followed by dispersion of potential clumps by passage through a 40 µm cell strainer (Corning, Durnham, NC).

Finally, primary CGC and MGC were plated in 12-well plates (Corning, Kennebunk, ME) at a density of 300 000 cells/well and were left to attach for two days in basal medium (Dulbecco's Modified Eagle

Medium/Nutrient Mixture F-12 [Gibco, Paisley, UK] supplemented with 10% fetal calf serum [FCS] [Lonza, Verviers, Belgium] and 1% penicillin/streptomycin/amphotericin B [Lonza, Walkersville, MA]) at 37 ^{o}C and 5% CO_2. The medium was refreshed after two days and then daily until the culture was devoid of red blood cells upon visual inspection (on average four days). Thereafter, the cells were cultured for 63 hours in medium with either 0.34 mmol/L LDL cholesterol, 0.34 mmol/L HDL cholesterol or the equivalent volume of phosphate-buffered saline (PBS; Gibco, Paisley, UK). FCS was not added to this medium as it contains BA. LDL and HDL were isolated by sequential ultracentrifugation ($1.019 < d < 1.063$ and $1.063 < d < 1.21$, respectively) from plasma of healthy male volunteers. HepG2 cells (a hepatoma cell line) and empty wells were used as positive and negative controls for each condition, respectively.

Bile acid measurements

In blood and FF, measurement of total BA were conducted using an enzymatic fluorimetric assay and by liquid chromatography-mass spectrometry (LC-MS) as previously described [8]. In cell culture supernatants and cell lysates, BA were measured by LC-MS as previously described [8].

Protein measurement

Total protein levels were measured in matched FF and serum using the BCA Protein Assay Kit (Pierce, Rockford, IL) following the instructions provided by the manufacturer.

Isolation of RNA and measurement of mRNA levels by quantitative real-time PCR

Total RNA was obtained from freshly isolated MGC and CGC and from healthy human livers using TRI Reagent (Sigma, St. Louis, MO) and quantified with a Nanodrop spectrophotometer (Nanodrop

2000c, Thermo Scientific). Complementary DNA was synthesized from 1 µg of RNA using Moloney-Murine Leukemia Virus reverse transcriptase (Invitrogen, Carlsbad, CA). Quantitative real-time PCR analysis was performed on a Real-Time PCR system (StepOnePlus, Applied Biosystems). Primers and fluorogenic probes (Table 1) were designed with the Primer Express software (Thermo Fisher Scientific) and synthesized by Eurogentec (Seraing, Belgium). Gene expression levels were normalized to the housekeeping gene peptidylprolyl isomerase G and calculated using the delta-Ct method.

Western Blotting

Western blots for Na^+/Taurocholate Cotransporting Polypeptide (NTCP), apical sodium-dependent bile acid transporter (ASBT), ATP-Binding Cassette Sub-Family C Member 4 (ABCC4) and ATP-Binding Cassette Sub-Family C Member 3 (ABCC3) were performed on primary human MGC and CGC pooled from several patients. Protein was resolved by SDS-PAGE electrophoresis and subsequently blotted onto nitrocellulose (Trans-Blot Turbo Transfer Pack, Biorad, Hercules, CA). The proteins were visualized using NTCP antibody (kindly provided by Prof. Dr. Bruno Stieger, University Hospital Zurich, Zurich, Switzerland; raised in rabbit; dilution 1:500), ASBT antibody (kindly provided by Prof. Paul A. Dawson, Wake Forest University School of Medicine, Winston-Salem, NC; raised in rabbit; dilution 1:10000), commercially available ABCC4 antibody (M4I-10, NBP1-42339, Novus Biologicals, MN; raised in rat; dilution 1:200) and commercially available ABCC3 antibody (sc-5776, C-18, Santa Cruz Biotechnology, Santa Cruz, CA; raised in goat; dilution 1:500), followed by the appropriate HRP-conjugated secondary antibody (goat anti-rabbit [P0448, Dako, Heverlee, Belgium], rabbit anti-goat [P0449, Dako, Heverlee, Belgium], and goat anti-rat [HAF005, R&D Systems, MN]).

Immunohistochemistry

Anonymized ovarian tissue from carriers of the BRCA mutation who underwent preventive oophorectomy was used. The tissue was fixed in formalin, embedded in paraffin, and sectioned into 3 µm slides. Prior to staining, paraffin was removed by immersion of the slides in xylol solution, followed by washing in ethanol. For antigen retrieval, either magnesium citrate (ASBT and ABCC4), EDTA (NTCP), or Tris/EDTA buffer (ABCC3) was used. To reduce endogenous peroxidase activity, the slides were incubated in hydrogen peroxide solution for 30 minutes. The slides were then incubated for one hour at room temperature in primary antibody dissolved in 1% BSA/PBS. The following dilutions were used: NTCP 1:500, ASBT 1:200, ABCC4 1:100 (see above), and ABCC3 1:100 (sc-5774, H-16, Santa Cruz Biotechnology, Santa Cruz, CA; raised in goat). Thereafter, they were incubated with secondary and tertiary goat anti-rabbit antibodies (P0448, Dako, Heverlee, Belgium), rabbit anti-goat (P0449, Dako, Heverlee, Belgium), and rabbit anti-goat antibodies (P0450, Dako, Heverlee, Belgium) diluted 1:100 in 1%BSA with 1% corresponding serum solution prepared in PBS. The order of these antibodies was determined by the nature of the primary antibody. The slides were then incubated for 10 minutes in 3,3'-diaminobenzidine and counterstained with hematoxylin. Finally, the slides were dehydrated and mounted.

Statistical analysis

Results of the measurements of BA in FF and matched serum and of measurements of total protein in FF and serum were expressed as median [interquartile range]. Their correlations were expressed as Spearman's r. For BA measurements in matched FF and serum, the levels were compared statistically using multilevel generalized estimating equations and the results were presented as odds ratio (95% confidence interval)). For protein measurement in matched FF and serum, the levels were compared statistically using Wilcoxon Signed Ranks test. Gene expression levels in CGC and MGC were compared statistically using the independent samples *t*-test if the values were normally distributed or the Mann-Whitney U test if the values were not normally distributed. A *P*-value <0.05

was considered statistically significant. Analyses were conducted using SPSS version 23 (SPSS, Inc., Chicago, IL).

Results

Passive diffusion of bile acids from blood into follicular fluid

Total bile acid (TBA) levels were measured in FF and matching serum from 142 MNC-IVF procedures corresponding to 131 unique patients. A summary of cycle characteristics can be found in Supplementary Table S1. There was a weak positive correlation between TBA in serum and that in blood (r_s=0.186, *P* = 0.027; Fig 1), and secondary bile acids were present in FF (deoxycholic acid derivatives [DCA]: 0.72 [0.39 to 1.19] µmol/L, n=139). Nonetheless, TBA levels were consistently about two-fold higher in FF compared to matching serum samples (10.10 [8.38 to 11.93] versus 5.89 [4.15 to 7.88] µmol/L, OR 58.01 (31.05 to 108.40), *P* < 0.001; Fig. 2) and primary BA were more abundant in FF than in matched serum (mean of 73% versus 60% of total BA, *P* < 0.001).

To study whether the higher levels of FF BA may be related to a different protein composition of FF as compared to serum, total protein levels were measured in FF and matched serum samples from 10 individual patients for which total BA measurements were available. The levels of protein in FF were significantly lower than those in matched serum (56.16 [51.86 to 59.25] mg/mL versus 69.83 [67.54 to 76.32] mg/mL, *P* = 0.005). Moreover, there was no correlation between protein levels and total BA in FF (r_s=-0.006, *P* = 0.987).

Local ovarian bile acid production

To study whether BA are locally produced in the ovaries, mRNA expression of key genes involved in BA production and metabolism was measured in freshly isolated MGC (nine individual patients) and CGC (eight individual patients) (Fig 3). For the BA production enzymes, healthy human livers served

as positive control (n=4). Prior to all analyses on primary MGC and CGC, the presence of anti-Muellerian hormone type-2 receptor (*MISR-II*, markers specific for GC) and steroidogenic activity (ie, estrogen production upon induction with follicle stimulating hormone and androstenedione) of the isolated primary cells was confirmed (data not shown). Gene expression of *CYP7A1* could not be detected in either MGC or CGC, indicating that the classical pathway of BA production is not present. Very low level mRNA expression of *CYP8B1*, *CYP27A1,* and *CYP7B1* was inconsistently detected in MGC and CGC, also making it highly unlikely that there is a discernible, substantial *de novo* synthesis of BA via the alternative pathway in the ovary. Nonetheless, the nuclear receptors Farnesoid X Receptor (*FXR*), Retinoic Acid Receptor (*RXR*)-alpha, Liver X Receptor (*LXR*)-alpha, and Liver Receptor Homolog 1 (*LRH1*) as well as the G-Protein coupled bile acid receptor *TGR5* were present in both cell types (Figure 4, Supplementary Table S2). The level of mRNA expression of FXR, RXR-alpha, LXR-alpha, and LRH1 was hereby significantly higher in CGC than in MGC ($P = 0.023$ for *FXR*, $P < 0.001$ for all other).

To further formally test the concept of *de novo* synthesis of BA via the alternative pathway in the ovary, MGC and CGC were cultured in medium without or with added lipoproteins as a substrate for BA synthesis (0.34 mmol/L LDL or 0.34 mmol/L HDL). BA content of cell supernatants and lysates was measured after 63 hours. In over 10 individual experimental repeats, BA could never be detected in any appreciable amounts in GC cultures (medium and cells) but were consistently present, though in low amounts, in material from HepG2 cultures.

Active transport of bile acids from blood into follicular fluid

To explore the possibility of active transport of BA from blood into FF, mRNA expression studies of common BA importer (NTCP, ASBT, OATP1B1, OATP1B3) and exporter (Organic Solute Transporter Subunit alpha [OST-alpha], Organic Solute Transporter Subunit beta [abcb11

], ATP Binding Cassette Subfamily B Member 11 [ABCB11], ABCC3, ABCC4) proteins were performed in freshly isolated GC, harvested from FF from COH-IVF procedures. NTCP, ASBT, OST-alpha, *ABCC3*, and *ABCC4* transcripts were present at varying levels in MGC and CGC (Fig. 5, Supplementary Table S2). The level of gene expression of *ABCC3* was significantly higher in CGC as compared to MGC ($P < 0.001$). For NTCP, ASBT, OST-alpha, and *ABCC4* there was no significant difference in the levels of gene expression between the two granulosa cell types. OST-beta and *ABCB11* expression was below detection level in both cell types (data not shown). Since OST-alpha is only functional in the presence of OST-beta [21], protein expression of OST-beta was not studied.

Next, protein expression of the above-mentioned transporters was studied (Fig. 6). Freshly isolated MGC and CGC from multiple patients were pooled and protein expression was assessed by Western blotting. Immunohistochemistry was performed on paraffin-embedded human ovarian tissue. In tertiary follicles, the BA importers ASBT and NTCP were present in both theca and MGC, and NTCP was additionally detected in CGC. The organic anion exporter ABCC3 was present in theca cells, MGC and CGC. ABCC3 expression could not be confirmed by Western blot of MGC. The presence of the BA exporter ABCC4 was confirmed in Western blots of CGC and MGC, but not in immunohistochemical staining of ovarian tissue. Finally, in immunohistochemistry of primary and primordial follicles, NTCP and ABCC3 were present in GC, whereas ASBT was absent. Finally, all three transporters were also clearly detectable in the oocyte.

Discussion

The results of the present study suggest that BA present in FF in higher amounts as compared to serum likely reach the FF from the systemic circulation by passive diffusion and active transport. Conversely, in human ovarian granulosa cells, local BA production is highly unlikely to occur. To our knowledge, this is the first report of BA transporters being present in ovarian follicles.

The BA synthesis pathway is traditionally thought to be exclusively present in the liver. However, a previous microarray study in human GC from hyperstimulation-IVF suggested that components of the classical and alternative pathway of BA synthesis are present in human CGC [17]. In the current study, primary BA were two-fold more abundant in FF than in serum, indicating that local production may take place. Moreover, in partial agreement to previous work[17], mRNA expression of certain BA synthesis enzymes of the alternative pathway (namely *CYP27A1* and *CYP7B1*) was found, although only very weak and inconsistent. However, physiological relevance could not be confirmed, since GC did not produce BA in any of the applied conditions. In addition, mRNA expression of *CYP7A1*, the key enzyme of the classical pathway, was completely absent. Combined, these data make it highly unlikely that BA are being locally produced in the ovary. The cause of this discrepancy between previous results and ours is unclear, but it may be related to differences in culture conditions [17]. In the present study, FCS was not added to the culture medium, as this contains BA and may thus bias the results.

Instead of local synthesis, our results suggest that ovarian BA are being passively and actively transported into FF. To enter the FF, blood components must pass through the blood-follicular barrier (BFB) composed of, from the exterior to the follicle, a vascular wall (endothelium and basement membrane), theca cells, follicular basement membrane, and GC [1]. Passage of molecules from blood into FF is size- and charge-dependent [1]. BA are small compounds that are predominantly present in blood bound to albumin and lipoproteins [22]. With regards to the former, immunohistochemical studies have shown albumin to be present in the structures composing the BFB and the follicular antrum, suggesting that it passes unhindered from blood into FF [10]. As for the latter means of transport, high density lipoproteins are the sole type of lipoproteins present in FF [23]. Given their small size, they are assumed to originate from blood by diffusion across the BFB [24]. Indeed, there was a positive correlation between BA in FF and those in blood, supporting the concept of passive diffusion. This is further reinforced by the presence of secondary BA in FF, since secondary BA originate from the modification by intestinal bacteria, which are not expected to be present in FF.

In conclusion, BA are likely to pass unhindered through the BFB as cargo of serum proteins, such as albumin, and possibly also high density lipoproteins. However, the much higher levels of FF BA as compared to blood (which is in agreement with the work of Smith et al [17]) and the lack of a correlation between protein levels and BA levels in FF already suggest that facilitated transport, in addition to diffusion, takes place. The relative overabundance of BA importers (NTCP and ASBT) over exporters (ABCC3) found in the present study may explain BA accumulation in FF. In the present study, we have focused on the most common BA transporters and it cannot be excluded that other, less well-known importers and exporters may also be present in ovarian follicles. Since active transport is not formally shown in the current study, studies quantifying the relative transport capacities of each transporter in and out of FF would be interesting and additive to the current work. However, these are technically very challenging to perform and virtually impossible to carry out in humans. Finally, it should be mentioned that blood BA concentrations show diurnal variations, ie, are higher after ingestion of a meal. Since blood was collected under fasting conditions, high FF BA concentrations may in part reflect an earlier exposure to high serum BA concentrations.

Accumulation of BA in FF during oocyte development and the presence of BA transporters in the oocyte as well as of a signaling system responsive to bile acids in the follicular environment argue in favor of a biological function of ovarian BA in human reproduction. Over the past decade there has been extensive research indicating that BA are more than just mere detergents, and also fulfill important endocrine functions. Indeed, they are involved in the regulation of glucose and lipid metabolism, immunity, and gut microbiota function via nuclear receptors such as FXR and TGR5 [13]. Moreover, BA seem to be involved in fertility and programming of offspring health. Ursodeoxycholic acid derivatives in human FF are associated with the development of top quality embryos [8]. In animals, tauroursodeoxycholic acid has been shown to reduce apoptosis of mouse and pig embryos and to increase the implantation and live-birth rate in mice [25-27]. In male rodents, supplementation of the diet with cholic acid results in reduced fertility, altered sperm methylation patterns, and increased perinatal mortality as well as metabolic changes of the surviving pups, effects that seem to

be mediated by TGR5 [28, 29]. Despite emerging studies on the possible mechanistic function of BA in animals, knowledge on BA in human reproduction is scarce and difficult to obtain due to ethical concerns regarding research with gametes and embryos.

One of the strengths of this work is the study of two different types of human GC, CGC and MGC. Moreover, as to the best of our knowledge, we are the first to demonstrate the presence of the classic BA transporters NTCP and ASBT outside hepatocytes and ileocytes, respectively. However, the present study also has points that should be approached cautiously. Firstly, it cannot be excluded that the freshly isolated GC may have been contaminated with other types of cells (eg, vaginal epithelial cells, ovarian epithelial cells, ovarian stromal cells, theca cells) that may have affected the results of protein detection and would thus explain the discrepancies between the Western Blot and immunohistochemistry results. For example, cancerous epithelial ovarian cells have been previously shown to express *ABCC4* [30]. In GC cultures, however, the majority of cells stained positive for MISR-II, indicating that, at least in cultures, contamination with other cells was minimal. Secondly, the FF is naturally rich in steroid hormones. In the present study, gonadotrophins stimulating steroid hormone production were not added to the culture medium, which may alter the functioning of the GC. However, the steroidogenic capacity of the cultured cells was intact, indicating that the cultivated cells were vital.

In summary, the results of the present study suggest that BA likely reach ovarian follicles by both passive and active transport from the blood compartment. Further studies are warranted to gain insight into the regulation of BA transport from blood into FF and into a potential impact of dysfunctional transport on reproductive physiology. Moreover, changes in blood BA composition may possibly impact FF BA composition, which would enable lifestyle and pharmacological interventions to prevent and treat infertility.

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Figure legends

Figure 1. Relationship of total bile acid levels in follicular fluid and in matching blood. Solid black line represents the best fitting line for the relationship between total bile acids in blood and in follicular fluid. rs=0.186, $P = 0.027$.

Figure 2. Comparison of total bile acid levels in follicular fluid and in matching blood. ***$P < 0.001$.

Figure 3. mRNA expression of key enzymes for bile acid production in mural and cumulus granulosa cells and human livers. CGC – cumulus granulosa cells, MGC – mural granulosa cells.

Figure 4. mRNA expression of bile acid responsive receptors in mural and cumulus granulosa cells. CGC – cumulus granulosa cells. MGC – mural granulosa cells. *$P < 0.05$. ***$P < 0.001$.

Figure 5. mRNA expression of common transport proteins involved in the import (upper row) and export of bile acids (lower row). CGC – cumulus granulosa cells, MGC – mural granulosa cells. ***$P < 0.001$.

Figure 6. Protein expression of the bile acid importers NTCP and ASBT and the bile acid exporter ABCC3 in cell lysates of human primary cumulus and mural granulosa cells (upper row: Western blot) and in ovarian tissue (middle row: immunohistochemical staining of tertiary follicles, scale bar represents 500 µm; lower row: immunohistochemical staining of primordial and primary follicles, scale bar represents 50 µm). CGC – cumulus granulosa cells, MGC – mural granulosa cells.

Table 1. Primer sequences.

Gene name	Sequence
PPIG	Forward: 5'- TGGAGCCATGGGAATAAAGGT - 3'
	Reverse: 5'- CTCTTCCAGCAGGTTGATTGTTAAT - 3'
	Probe: 5' - CAACGTCCTCGATGTTTTTTTGACATTGCC – 3'
CYP7A1	Forward: 5' - TCAGCTTGGAAGGCAATCCTAT - 3'
	Reverse: 5' - AGCCTCAGCGATTCCTTGATTA - 3'
	Probe: 5' - CTGGCAGGTCATTCAGTTCTGCTTGACTC - 3'
CYP8B1	Forward: 5' - CCTGAGCTTGTTCGGCTACAC - 3'
	Reverse: 5' - TGCGGAACTCCATGAATAACTCTC- 3'
	Probe: 5' - CCTGTAGCAGGTCCTGCTCCTTGTCCTT - 3'
CYP27A1	Forward: 5' - TGCGGGCAGAGAGTGCTT - 3'
	Reverse: 5' - ACAGGATGTAGCAAATAGCTTCCA - 3'
	Probe: 5' - CAGGTGTCGGACATGGCTCAACTCTTCT - 3'
CYP7B1	Forward: 5' - CTTGAAATAGGAGCACATCATTTAGG- 3'
	Reverse: 5' - GATAATACATTGCCCAGAACATAGTTG - 3'
	Probe: 5' - CTCTGGGCCTCTGTGGCAAACACTATTC - 3'
FXR	Forward: 5' - AGGGGTGTAAAGGTTTCTTCAGGA- 3'
	Reverse: 5' - ACACTTTCTTCGCATGTACATATCCAT- 3'
	Probe: 5' - TTGCCCCCGTTTTTACACTTGTACACAGC- 3'
RXR-alpha	Forward: 5' - GCAAACATGGGGCTGAACC - 3'
	Reverse: 5' - GCTGCTTGGCAAATGTTGGT - 3'
	Probe: 5' - CAGCTCGCCGAACGACCCTGTC - 3'
LXR-alpha	Forward: 5' - CTTGCTCATTGCTATCAGCATCTT - 3'

	Reverse: 5’ - ACATATGTGTGCTGCAGCCTCT - 3’
	Probe: 5’ - TCTGCAGACCGGCCCAACGTG - 3’
LRH-1	Forward: 5’ - CAGAGAACTTAAGGTTGATGACCAA - 3’
	Reverse: 5’ - GGTAAATGTGGTCGAGGATTAAGAG- 3’
	Probe: 5’ - TCACTCCAGCAGTTCTGAAGCAGCTTCA - 3’
TGR5	Forward: 5’ - CGTCTACTTGGCTCCCAACTTC - 3’
	Reverse: 5’ - GGCCTCAGGACTGCCATGTA - 3’
	Probe: 5’ - CTCTCCCTGCTTGCCAACCTCTTGC - 3’
VDR	Forward: 5’ - CCGCATCACCAAGGACAAC - 3’
	Reverse: 5’ - TCATCTGTCAGAATGAACTCCTTCA - 3’
	Probe: 5’ - AGGCCTGCCGGCTCAAACGC - 3’
PXR	Forward: 5’ - ACATGCTGAAGAAGCTGCAGCT - 3’
	Reverse: 5’ - GGCGGTCTGGGGAGAAGA - 3’
	Probe: 5’ - ATGGCCTGCATCAGCACATACTCCTCC - 3’
CAR	Forward: 5’ - ACCGACCTGGAGTTACCCAGA - 3’
	Reverse: 5’ - CTTCGCATACAGAAACCGATCC - 3’
	Probe: 5’ - CTTTGCAGAGTCAGTGCCATCTCCTCTTG - 3’
FGFR4	Forward: 5’ - TGTGCAAGGTGTACAGCGATG - 3’
	Reverse: 5’ - TATTGATGTCTGCAGTCTTTAGGACTT - 3’
	Probe: 5’ - CGTCATCAACGGCAGCAGCTTCG - 3’
FGF19	Forward: 5’ - ATGCAGGGGCTGCTTCAGTA - 3’
	Reverse: 5’ - AGCCATCTGGGCGGATCT - 3’
	Probe: 5’ - TCCTCGAAAGCACAGTCTTCCTCCG - 3’
KLB	Forward: 5’ - AATGGCTGGTTCACAGACAGTC - 3’
	Reverse: 5’ - TCATCTAACCTTATTGCTTGAAGCA - 3’
	Probe: 5’ - ACCACGGCCATCTACATGATGAAGAATTTC - 3’

NTCP	Forward: 5’ - TGATATCACTGGTCCTGGTTCTCA - 3’
	Reverse: 5’ - GCATGTATTGTGGCCGTTTG - 3’
	Probe: 5’ - TCCTTGCACCATAGGGATCGTCCTCA - 3’
ASBT	Forward: 5’ - CACGCAGCTATGTTCCACCAT - 3’
	Reverse: 5’ - GAGCGGGAAGGTGAATACGA - 3’
	Probe: 5’ – CAGCTCTCCTTCACTCCTGAGGAGCTC A - 3’
OST-alpha	Forward: 5’ - GGTGAGCAGAACATGGGAGC - 3’
	Reverse: 5’ – ATGGAGGGCTGTAGGGCAGT - 3’
	Probe: 5’ - AAATTTGCTCTGTTCCAGGTTCTCCTCATCC - 3’
OST-beta	Forward: 5’ – CAGGAGCTGCTGGAAGAGAT - 3’
	Reverse: 5’ - GACCATGCTTATAATGACCACCA - 3’
	Probe: 5’ - CGTGTGGAAGATGCATCTCCCTGGAATCATTC - 3’
ABCB11	Forward: 5’ - ACATGCTTGCGAGGACCTTTA - 3’
	Reverse: 5’ – GGAGGTTCGTGCACCAGGTA - 3’
	Probe: 5’ - CCATCCGGCAACGCTCCAAGTCT - 3’
ABCC3	Forward: 5’ - GCCATCGACCTGGAGACTGA - 3’
	Reverse: 5’ - GACCCTGGTGTAGTCCATGATAGTG - 3’
	Probe: 5’ - CATCCGCACCCAGTTTGATACCTGCAC - 3’
ABCC4	Forward: 5’ - AAGTGAACAACCTCCAGTTCCAG - 3’
	Reverse: 5’ - GGCTCTCCAGAGCACCATCT - 3’
	Probe: 5’ - CAAACCGAAGACTCTGAGAAGGTACGATTCCT - 3’
OATP1B1	Forward: 5’ - AAGCCACTTCTGCTTCTGTGTTT - 3’
	Reverse: 5’ - AATTCTTAGTGAAAGGACCAGGAACT - 3’
	Probe: 5’ - CTCAAAAATAACATCTTACTGAATCAATGCA - 3’
OATP1B3	Forward: 5’ - AACATGTAATTTGGACATGCAAGAC - 3’
	Reverse: 5’ – TTGTCAGTGAAAGACCAGGAACA - 3’

Probe: 5’ - CTGCTGCCAACTAACATTGCATTGATTCATT - 3’